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Surface glycoproteins of influenza A H3N2 virus modulate virus replication in the respiratory tract of ferrets

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ABSTRACT

The hemagglutinin (HA) genes of the influenza A H3N2 subtype viruses isolated from 1968 to 2010 have evolved substantially but their neuraminidase (NA) genes have been relatively less divergent. The H3N2 viruses isolated since 1995 were found to replicate in the lower respiratory tract of ferrets less efficiently than the earlier isolates. To evaluate whether the HA or/and NA or the internal protein gene segments of the H3N2 virus affected viral replication in the respiratory tract of ferrets, recombinant A/California/07/2004 (CA04) (H3N2) virus and its reassortants that contained the same CA04 internal protein gene segments and the HA and/or NA of A/Udorn/309/1972 (UD72) or A/Wuhan/359/1995 (WH95) H3N2 viruses were generated and evaluated for their replication in the respiratory tract of ferrets. All the reassortant viruses replicated efficiently in the upper respiratory tract of ferrets, but their replication in the lower respiratory tract of ferrets varied. In contrast to the UD72-HA reassortant virus that replicated efficiently in the lungs of ferrets, the virus with the WH95-HA or the CA04-HA either replicated modestly or did not replicate in the lungs of ferrets. The reassortants with the WH95-HA and UD72-NA or CA04-NA had the tendency to lose a N-linked glycosylation site at residue 246 in the HA, resulting in viral lung titer of 100-fold higher than the virus with the HA and NA from WH95. The UD72-NA had the highest neuraminidase activity and increased viral replication by up to 100-fold in tissue culture cells during early infection. Thus, our data indicate that both the HA and NA glycoproteins play important roles in viral replication of the H3N2 influenza virus in ferrets.

Introduction

Influenza A viruses are significant human pathogens that cause annual epidemics and occasional influenza pandemics (Molinari et al., 2007). The hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins of influenza virus are essential for viral entry into target cells and release of progeny viruses from infected cells, respectively. The HA is the major antigenic glycoprotein that induces neutralizing antibodies. The HA globular head possesses the receptor-binding sites (RBS) that mediate viral binding to the galactose-linked sialic acid receptors on the cell surface. The HA proteins of human influenza viruses preferentially recognize sialic acids linked (SAL) via $\alpha-2,6$ glycosidic linkage while the avian viruses preferentially bind to $\alpha-2,3$ SAL. A number of amino acids in the HA have been shown to be critical for preferential recognition of $\alpha-2,6$ SAL or $\alpha-2,3$ SAL receptors and are implicated in modulating viral virulence in the hosts (Martin et al., 1998; Matrosovich et al., 2000; Nakajima et al., 2003; Suzuki et al., 2000). In particular, changes at residues 226 and 228 in the HA of human influenza A/Udorn/307/1972 (H3N2) virus switched its receptor recognition specificity from $\alpha-2,6$ to $\alpha-2,3$ SAL, which enabled this human H3N2 virus to replicate in the intestine of duck in which $\alpha-2,3$ SAL receptors are abundant (Vines et al., 1998). Since the emergence of H3N2 viruses in humans in 1968, the HA molecule has undergone substantial antigenic changes (antigenic drift) to escape neutralization of antibodies from the host, either from prior infection or vaccination. The antigenic drift could be the accumulation of amino acid substitutions in or surrounding the antigenic sites, RBS and/or N-linked glycosylation sites in the HA (Nakajima et al., 2003).

Glycosylation on the globular head of the HA may shield RBS or modify receptor recognition specificity (Ohuchi et al., 1997). The number of the N-linked glycosylation sites in the globular head of the H3 HA1 region has increased from two in the 1968 strains to six or seven sites among the contemporary strains (Abe et al., 2004; Seidel et al., 1991; Zhang et al., 2004). The N-linked glycosylation at residues 126, 135, 144, 165 and 246 of the H3 HA has been found to be associated with decreased virulence in mice, which is characterized by reduced viral replication in the lungs and pulmonary inflammation (Reading et al., 2009; Vigerust et al., 2007; Wagner et al., 2002).

The NA is a receptor-destroying enzyme that cleaves the sialic acid from the budding virions to facilitate viral release from infected cells and to prevent virion aggregation. The functional

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balance between the HA and NA proteins are critical for virus growth, transmission and adaptation in the human host (Gulati et al., 2005; Ilyushina et al., 2005; Yen et al., 2011). The changes in the HA receptor binding activity are normally accompanied by concomitant changes in the NA to maintain a functional balance between the receptor binding and receptor destroying activities of these two proteins (Baum and Paulson, 1991; Kaverin et al., 1998; Wagner et al., 2000). We previously reported that replication of influenza A/Fujian/411/2002 (H3N2) in eggs and MDCK cells could be improved by either changing two HA residues to increase the HA's receptor-binding ability or by changing two NA residues to lower the NA's enzymatic activity (Lu et al., 2005).

Ferrets are considered as a suitable small animal model for evaluating influenza virus pathogenesis because of their susceptibility to human influenza virus infection and their disease symptoms that are similar to humans (Matsuoka et al., 2009). Ferrets and humans also share similar anatomy in lung physiology, airway morphology, cell types and distribution of the $\alpha-2,6$ and $\alpha-2,3$ SAL receptor in the respiratory tract (Xu et al., 2010). Ferrets have been proved to be instrumental in evaluation of vaccine mediated protection (Subbarao and Luke, 2007). We found that *wt* H3N2 viruses isolated since 1995 do not replicate well in the lower respiratory tract (lungs) of ferrets compared to the earlier strains. To examine the molecular determinants required for H3N2 viral replication in ferret lungs, representative strains isolated from the past decades were examined. Using reassortant viruses generated by reverse genetics (Jin et al., 2003), we evaluated the contribution of the HA and NA to the replication of the H3N2 virus in the respiratory tract of ferrets.

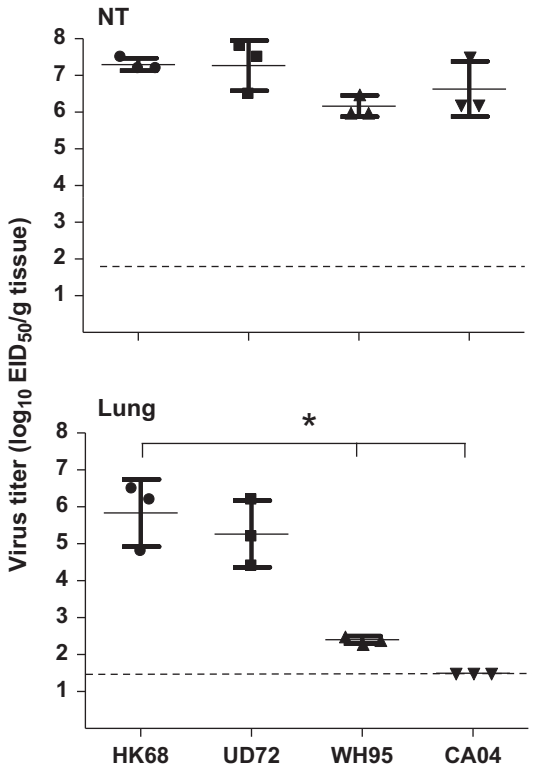

Fig. 1. Replication of H3N2 viruses in the ferret respiratory tract. Groups of three ferrets were infected with 10^7 FFU of each of the following H3N2 *wt* viruses: HK68, UD72, WH95 and CA04. Infectious virus titers in nasal turbinates (NT) and lung homogenates were determined on day 3 post-infection by EID_{50} assay. Dashed line indicates the detection limit of 1.5 $\log_{10}$ EID_{50} per gram of tissue. $^*P < 0.05$, one way ANOVA test.

Results

Replication of the H3N2 viruses in the respiratory tract of ferrets

Representative H3N2 viruses isolated from 1968 to 2004 (HK68, UD72, WH95, and CA04) were evaluated for their replication efficiency in the respiratory tract of ferrets (Fig. 1). All four viruses replicated efficiently in the nasal turbinates (NT) and reached titers in the range of 6.0–7.8 $\log_{10}$ EID_{50}/g tissue on day 3 post-infection. In the lungs, HK68 and UD72 replicated most efficiently, reaching titers of 5.8 and 5.3 $\log_{10}$ EID_{50}/g, respectively. WH95 replicated to a titer of 2.3 $\log_{10}$ EID_{50}/g that was about 1000-fold lower than HK68 or UD72 while CA04 replication was not detected in the lungs of infected ferrets. These data demonstrated that more recent H3N2 viruses replicated less efficiently in the lower respiratory tract of ferrets compared to the earlier isolates.

Sequence alignment of the HA1 region of the H3 HA of these four viruses revealed that the HA protein sequences were substantially different from each other (Fig. 2A). Some of these differences occurred in or near the antigenic sites A and B, most likely due to the antigenic drift of these H3N2 viruses in the past four decades. The residues known to be critical for SAL receptor binding specificity of influenza A viruses (145, 186, 190, 225, 226, and 228) (Connor et al., 1994; Glaser et al., 2005) were also different among these four strains. In addition, the number of glycosylation sites in the HA globular head region evolved from 2 in HK68 and UD72, 4 in WH95, and up to 7 sites in CA04 (Fig. 2B and C). WH95 lost the glycosylation site at residue 81 but gained three new sites at residues 63, 126 and 246. CA04 gained three additional sites at residues 122, 133 and 144.

Contribution of the HA and NA to H3N2 virus replication in ferret lungs

To assess the relative contribution of the surface and internal protein gene segments to H3N2 virus replication in ferret lungs, reassortant viruses were generated using the same set of internal protein gene segments from CA04, while the HA and NA protein gene segments were derived from either UD72, WH95 or CA04. The reassortant viruses were evaluated for their ability to replicate in the respiratory tract of ferrets. As shown in Fig. 3A upper graph, the viruses containing homologous HA and NA of UD72, WH95 or CA04 replicated to titers in the range of 5.0–6.5 $\log_{10}EID_{50}$/g in the upper respiratory tract (nasal turbinates, NT). In the lungs, the virus with the HA and NA from UD72 replicated to a titer of 5.5 $\log_{10}$ EID_{50}/g, which was about 1000-fold higher than the titer of the virus with the HA and NA from WH95 (Fig. 3A, lower graph). Similar to the biological CA04 *wt* virus, replication of recombinant CA04 virus was not detected in the lungs. These data demonstrated that the internal protein gene segments of CA04 could support efficient viral replication of other H3N2 viruses in the ferret lungs and that the difference in viral replication was mainly determined by the HA and NA surface glycoproteins.

To further dissect the roles of the HA and NA in supporting H3N2 viral replication in the lungs of ferrets, reassortant viruses bearing homologous or heterologous HA and NA (Fig. 3A) were also generated and evaluated in ferrets. In the upper respiratory tract (Fig. 3A upper graph), the virus with CA04-HA paired with UD72-NA and UD72-HA paired with CA04-NA had a lower level of replication in the NT. In contrast, UD72 NA but not the CA04-NA slightly increased replication of the virus with WH95-HA. In the lower respiratory tract (Fig. 3B, lower graph), the reassortant virus with CA04-HA and UD72-NA did not replicate in the lungs of the infected ferrets; in contrast, the reassortant virus with UD72-HA and CA04-NA replicated to 5.3 $\log_{10}$ EID_{50}/g, indicating that the HA played a critical role in determining viral replication in the lungs. Interestingly, the reassortant virus with WH95-HA and

A

```
      61         71         81         91         101        111        121
HK68  GIDCTLIDAL LGDPHCDVFQ NETWDLFVER SKAFSNCYPY DVPDYASLRS LVASSGTLEF ITEGFTWTGV
UD72  -ID------- -------G-- ---------- ---------- ---------- ---------- IS-G-T----
WH95  -EN------- -------G-- -K-------- ---Y------ ---------- ---------- TN-G-N----
CA04  -KN------- ----Q--G-- -KK------- ---Y------ ---------- ---------- NN-S-N----

      131        141        151        161        171        181        191
HK68  TQNGGSNACK RGPGSGFFSR LNWLTKSGST YPVLNVTMPN NDNFDKLYIW GVHHPSTNQE QTSLYVQASG
UD72  T-N-G-NA-- -GPDSG---- ----YKSGST ---------- -DN------- -----S-DQE ----------
WH95  A-D-T-YA-- -GSVKS---- ----HKLEYK ---------- -DK------- -----S-DSD ----------
CA04  T-N-T-SS-- -RSNNS---- ----THLKFK ---------- -EK------- -----G-NND ----------
            A                     B                              B
      201        211        221        231        241        251        261
HK68  RVTVSTRRSQ QTIIPNIGSR PWVRGLSSRI SIYWTIVKPG DVLVINSNGN LIAPRGYFKM RTGKSSIMRS
UD72  ---------- ---------- -W--GLS--- ---------- -I-V---N-- ---------M ----------
WH95  ---------- ---------- -W--GIS--- ---------- -I-L-N-T-- ---------I ----------
CA04  ---------- ---------- -R--DIP--- ---------- -I-L-N-T-- ---------I ----------
```

B

Virus	Glycosylation sites in HA1								
	GS	63	81	122	126	133	144	165	246
HK 68	2		+					+	
UD72	2		+					+	
WH95	4	+			+			+	+
CA04	7	+		+	+	+	+	+	+

C

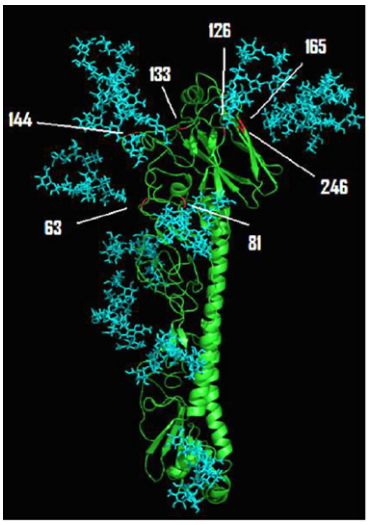

Fig. 2. Comparison of HA sequences of H3N2 viruses. (A) Alignment of HA protein sequences from amino acid 61 to 270. "–" indicates conserved residues; A and B indicate antigenic sites; residues involved in SAL receptor binding are underlined; N-linked glycosylation sites are in red. (B) Comparison of the glycosylation sites in HA1 of H3N2 viruses. GS: number of glycosylation site; "+" indicates potential GS at the indicated position. (C) H3 HA monomer with potential GS indicated was modified from the crystal structure of A/Aichi/2/68 using PyMOL (The PyMOL Molecular Graphics System, version 1.3; Schrödinger, LLC).

UD72-NA replicated to a titer of 5.0 $\log_{10}$ EID_{50}/g, which was more than 100-fold higher than that bearing WH95-HA and WH95-NA or WH95-HA and CA04-NA, suggesting that the NA also impacted viral replication. To determine whether the viruses recovered from the lungs of ferrets had any sequence changes in the HA and NA, the HA and NA genes of the lung isolates were sequenced. The HA of the reassortant virus with WH95-HA and UD72-NA had the change from glutamine to serine at residue 246 (N246S) in two out of the three isolates, the HA of the reassortant with WH95-HA and CA04-NA had the change from glutamine to arginine at residue 246 (N246R) in one of the isolates. None of the viruses had sequence changes in the NA gene. The changes at residue 246 resulted in the loss of a potential glycosylation site in the WH95-HA. This glycosylation site (Fig. 2A and B) appeared in the HA of the H3N2 viruses in 1980 and has since been maintained in most contemporary H3N2 viruses. The loss of the glycosylation site at residue 246 has been recently reported to be associated with increased viral replication of A/Beijing/353/1989 (H3N2) virus in mice by approximately 10-fold higher titer (Reading et al., 2009).

Effect of the glycosylation site on viral replication in ferrets

To further assess the role of the N246 glycosylation site on viral replication in ferret lungs, the N246S mutation was introduced into the HA of WH95 (WH95-HA N246S) and CA04 (CA04-HA N246S). Reassortant viruses that contained the HA with the N246S mutation and the homologous or heterologous NA were generated. The viruses containing HA N246S replicated to titers at 4–5 $\log_{10}EID_{50}$/g in the NT (Fig. 3B, upper graph). The efficient viral lung replication of the WH95-HA N246S reassortants was also independent on the NA, all three reassortants replicated at levels of 4.5–5.5 $\log_{10}EID_{50}$/g (Fig. 3B, lower graph). Relative to the virus with the HA and NA from WH95 with titer of approximately 2.5 $\log_{10}EID_{50}$/g in the lungs (Fig. 3A), the N246S mutation increased viral lung replication by approximately 100-fold. However, the reassortant viruses containing CA04-HA N246S and the NA from CA04, WH95, or UD72 did not replicate in the lungs (Fig. 3B and data not shown). The loss of the N246 glycosylation site was not sufficient for CA04 strain to replicate in the lungs. There are a total of 31 amino acids differed between the HA1 of WH95 and CA04, and their impact on viral replication was not investigated. Glycosylation of the HA N246 site was confirmed by Western blot analysis using a polyclonal antibody against H3 HA (Fig. 3C). The HA protein that had the N246S change migrated faster than the one without the change.

Effect of the NA on virus replication in MDCK cells

Since the NA protein is critical for influenza virus budding and spread in infected cells, WH95 reassortant viruses that contained WH95-HA and the NA of UD72, WH95 or CA04 were compared for their plaque morphology. As shown in Fig. 4A, heterologous NA did not significantly affect viral plaque morphology in MDCK cells. The single-step (MOI of 5.0) and multiple-step (MOI of 0.01) growth kinetics of these viruses in MDCK cells were examined (Fig. 4B and C). At MOI of 5.0, the virus with WH95-HA and UD72-NA grew to a titer of more than 10-fold higher than those bearing CA04-NA or WH95-NA at 8 h p.i.; no titer differences were observed at later time points (data not shown). At MOI of 0.01, the virus with UD72-NA also replicated to a titer that was 15-fold higher than the others at 24 h p.i., although the titer differences were not statistically significant at 48 and 72 h p.i. These data suggested that the UD72-NA may facilitate faster viral release than WH95-NA and CA04-NA.

The NA activities of these three WH95 reassortant viruses were examined. As shown in Fig. 5A, UD72 virus possessed greatest NA enzymatic activity that was about 1.9- and 1.3-fold higher than that of WH95 and CA04, respectively. The HA and NA protein contents in the purified viruses were examined by gel electrophoresis on SDS-PAGE and found to be comparable. The HA to NA ratio was about 4–5:1 as quantitated by ImageQuant TL. Thus, the observed differences in the NA activity reflected the difference in their enzymatic activity rather than difference in the amount of NA incorporation into virions.

The NA enzymatic activity of the three NA molecules was further examined using transiently expressed NA proteins. As shown in Fig. 5B, the enzymatic activity of UD72-NA was approximately 3.7- and 2.5-fold higher than that of WH95-NA and CA04-NA, respectively. Thus, the data obtained from

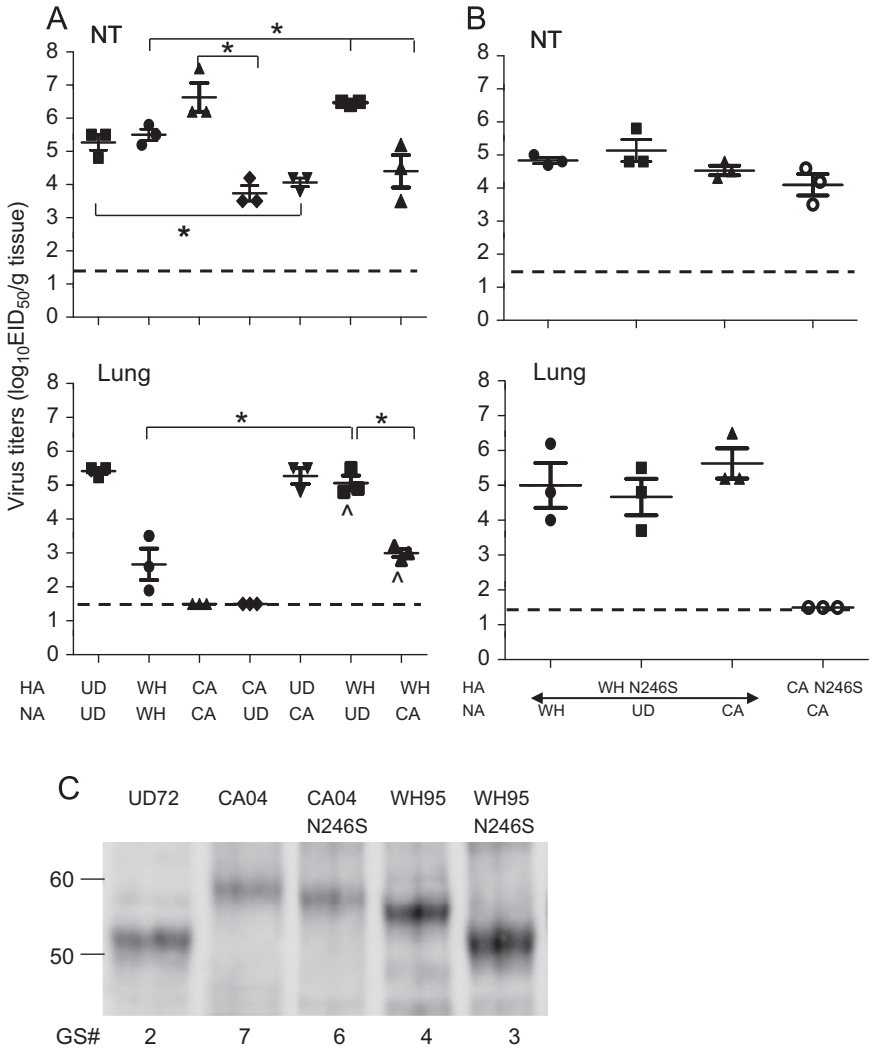

Fig. 3. Contribution of glycoproteins to viral replication in the respiratory tract of ferrets. Groups of three ferrets were infected with 10^7 FFU of virus bearing the same internal protein gene segments of CA04 with the indicated HA (A) or WH95 HA with N246S change (B) and the NA gene segments of UD72 (UD), WH95 (WH), or CA04 (CA). Infectious virus titers in nasal turbinates (NT) and lung homogenates were determined on day 3 post-infection by EID_{50} assay. Dashed line indicates virus detection limit of 1.5 $\log_{10}$ EID_{50} per gram of tissue. The horizontal line and error bar represent mean virus titer and standard derivation (SD), respectively. $^*P < 0.05$, one way ANOVA or *t* test. (C) Evaluation of HA glycosylation status of reassortant H3N2 viruses. The electrophoretic mobility of purified viruses was determined by Western blotting following electrophoresis on an 8–16% SDS-PAGE gel using anti-H3 antibody. Molecular size markers (kDa) are indicated on the left of the gel.

transiently expressed NA was consistent with the data obtained from the viruses, confirming that the UD72-NA protein possessed the highest neuraminidase activity.

Effect of the N246 glycosylation on viral antigenicity

The acquisition of oligosaccharides in the HA of H3N2 viruses has been considered as a mechanism to shield antigenic sites to allow the virus to escape immune pressure in humans (Abe et al., 2004). To examine if the loss of the N246 glycosylation site in the HA of WH95 and CA04 was associated with antigenic changes, ferrets were immunized with UD72, WH95 and CA04 *wt* viruses and postinfection sera were examined for their cross reactivity by the microneutralization assay (Table 1). UD72, WH95, and CA04 postinfection sera had high neutralizing antibody titers against each homologous virus but cross-reacted poorly to the heterologous viruses because they were antigenically distinct. UD72 postinfection ferret sera had lower reactivity to the earlier HK68 strain and much lower reactivity to WH95 and CA04. In contrast, WH95 and CA04 postinfection sera did not react with any of the earlier strains, WH95 also had lower reactivity to CA04. Both WH95 and CA04 cross-reacted well with their respective N246S mutants, with a titer difference of 2-fold compared to the virus with the N246 glycosylation site. Thus, the loss of the N246 glycosylation site in the HA of WH95 and CA04 had minimal impact on their antigenicity.

Discussion

In this study, we report that the early H3N2 influenza virus isolates replicated more efficiently in ferret lungs than the contemporary viruses. Since the molecular determinants of influenza viral replication in ferret lungs could be polygenic, we compared the replication of several reassortant H3N2 viruses that shared the same set of the internal protein gene segments to eliminate their potential confounding influence. The internal protein gene segments have been shown to play pivotal roles in viral replication and pathogenesis in mice and ferrets (Imai et al., 2010; Watanabe et al., 2009). This strategy allowed us to focus on understanding the contribution of the HA and NA proteins to H3N2 virus replication in the lower respiratory tract of ferrets.

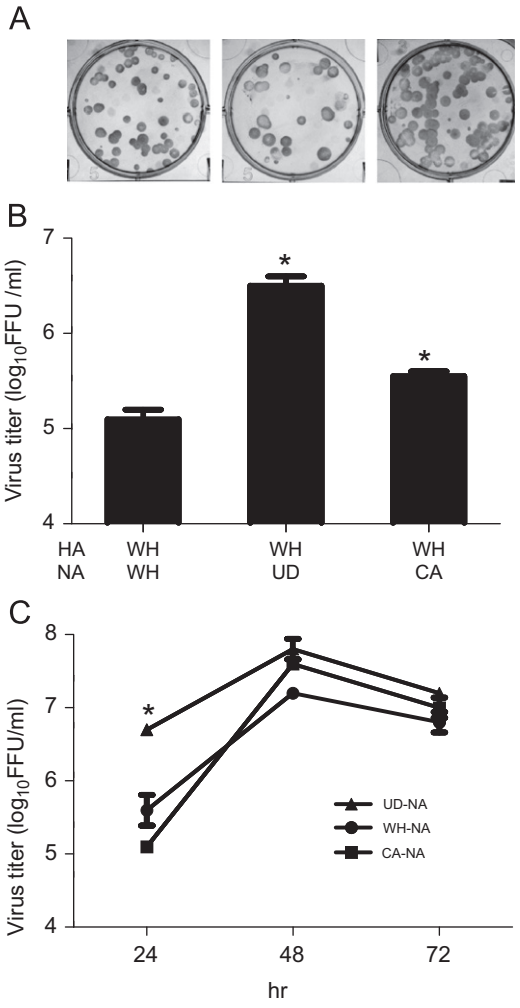

Fig. 4. Replication of H3N2 reassortant viruses in MDCK cells. (A) Plaque morphology of the indicated viruses in MDCK cells. Plaques were visualized by immunostaining at day 3 p.i. (B) MDCK cells were infected with the indicated virus at MOI of 5.0. The culture supernatants were collected at 8 h p.i. and viral titers were determined by FFA assay. (C) MDCK cells were infected with the indicated virus at MOI of 0.01. The culture supernatants were collected at 24, 48 and 72 h p.i. and viral titers were determined by FFA assay. $^{*}P < 0.05$, one way ANOVA test.

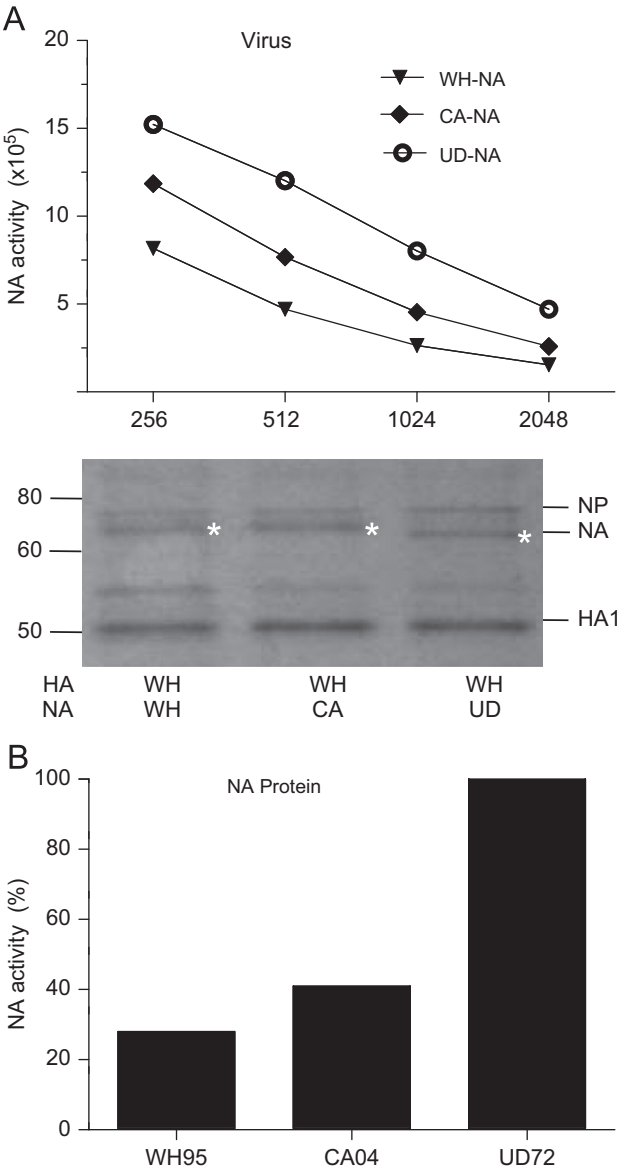

Fig. 5. Comparison of the NA activities of different H3N2 viruses. (A) The same amount of purified H3N2 virus was 2-fold serially diluted and examined for NA activity. The data represents example of three independent experiments. The relative amount of the viral proteins was examined by gel electrophoresis followed by Coomassie blue staining. The intensity of the HA and NA bands were quantitated by ImageQuant TL. The NA protein is indicated by *. Molecular size markers (kDa) are indicated on the left of the gel. (B) BSR-T7 cells were co-transfected with plasmids encoding N2 protein or Firefly luciferase. At 48 h post-transfection, cells were lysed, 2-fold serially diluted; aliquots were analyzed for NA or luciferase activities. The NA activity ($OD_{460\ nm}$) was normalized with luciferase activity ($OD_{482\ nm}$) and expressed as relative NA activity. The data represent one of the three independent experiments.

Our data clearly demonstrated that the HA is a key determinant in supporting efficient H3N2 viral replication in the lungs of ferrets; removal of a glycosylation site at residue 246 from the globular head of WH95-HA enhanced viral replication.

The difference in virulence of the H3N2 viruses were previously examined in a mouse study where reassortant viruses bearing the surface glycoproteins of A/Hong Kong/1/1968 caused more severe disease than those of A/Panama/2007/1999 (Vigerust et al., 2007). However, such work has not been systematically studied in the ferret model, which is commonly used for studying influenza viruses for viral pathogenesis, transmission and vaccine mediated protection. In this study, we found that replication of different H3N2 viruses in the ferrets varies substantially due to their HA and NA. Chen et al. (2008) previously reported that A/Wyoming/2/2003 (H3N2) failed to replicate in the lungs of mice and was not pathogenic (MLD_{50} of $> 10^6$ PFU); however, a reassortant virus with the HA and NA genes from A/Thailand/16/2004 (H5N1) allowed the virus to replicate to high titers in the lungs of mice. A reassortant virus that possessed the HA and NA genes from a swine H3N2 virus (A/swine/MN/593/1999) made the virus more pathogenic in pigs (Landolt et al., 2006).

HA is a receptor binding protein that mediates viral entry. Alteration of the HA sequence at the HA cleavage site, receptor binding or glycosylation site could modulate viral virulence (Deshpande et al., 1987; Klenk et al., 2002; Vigerust et al., 2007). The HA genes of the H3N2 viruses used in this study had changes in the key receptor binding residues (Connor et al., 1994; Martin et al., 1998). HK68, UD72, WH95 and CA04 differed at the known receptor binding region. HK68 and UD72 have E190 and L226, while WH95 and CA04 have D190 and I226. The 190 and 226 residues have been reported to be critical for preferentially recognizing $\alpha-2,3$ and $\alpha-2,6$ SAL, respectively (Pekosz et al., 2009; Vines et al., 1998). Using sialic acid specific chicken

Table 1
Antigenicity of the H3N2 reassortants[a].

Test virus	Neutralizing Ab titers against indicated viruses					
	UD72		WH95		CA04	
	Serum #1	Serum #2	Serum #1	Serum #2	Serum #1	Serum #2
HK68	160	320	≤20	≤20	≤20	≤20
UD72	**640**	**1280**	≤20	≤20	≤20	≤20
WH95	20	20	**1280**	**2560**	≤20	≤20
WH95 N246S	40	20	1280	1280	≤20	20
CA04	40	80	80	80	**1280**	**2560**
CA04 N246S	40	80	80	80	640	1280

[a] Groups of two ferrets were infected with 10^7 FFU of *wt* UD72, WH95, or CA04 virus intranasally. Serum samples were collected on day 28 p.i. and tested for their neutralizing antibody titers against homologous (bold and underlined) and heterologous viruses using microneutralization assay.

erythrocytes (Nobusawa et al., 2000) to examine viral receptor binding specificity, HK68, UD72, WH95 and CA04 and the N246S variants exhibited similar binding to α−2,6 SAL. However, Their binding to α−2,3 SAL were different. HK68 and UD72 had higher binding to α−2,3 SAL, WH95 and CA04 had lower binding to α−2,3 SAL (data not shown). It is possible that viral binding difference to α−2,3 SAL may contribute to their lung replication in ferrets, but their differential replication in ferret lungs may not be simply explained by their receptor binding difference.

The H3N2 viruses have evolved to gain a number of glycosylation sites in the HA globular head region since they entered human population in 1968. The addition of carbohydrates to the HA of recent H3N2 viruses could decrease viral replication by reducing receptor binding activity and mask antigenic sites from recognition by antibody resulting in antigen drift (Abe et al., 2004). The acquisition of the glycosylation sites at residues 63, 126, 165 and/or 246 in the HA of A/Beijing/359/1989 (BJ89, H3N2) has been reported to be associated with reduced H3N2 viral replication in the lungs of mice (Tate et al., 2011). BJ89 lacking glyscoysylation at 165 and/or 246 was less sensitive to neutralization by murine collectins and was more virulent in mice. In the present study, we found that the removal of the N246 glycosylation site from the HA increased WH95 replication in the lungs of ferrets, but not the replication of CA04. It is possible that the collection-like substance in the respiratory tract of ferrets could neutralize virus with less glycosylation moiety such as WH95-HAN246S more efficiently than the virus with additional glycosylation sites on the HA such as CA04 virus. Glycosylation acquisition is one of the mechanisms for influenza virus to escape immune defense in the respiratory tract in the humans. The addition of glycosylation at residue 246 in the HA of A/Aichi/2/1968 (Aichi68) (H3N2) resulted in reduced receptor binding affinity and lower reactivity with Aichi68 infected human sera (Abe et al., 2004). However, the effect of the N246 glycosylation on antigenicity of WH95 and CA04 was not significant as examined by WH95 and CA04 immunized ferret sera, which might be more apparent by two-way cross reactivity assay including WH95 N246S and CA04 N246S immunized ferret sera.

The HA receptor binding activity has to be balanced with the NA enzymatic activity in order to achieve efficient replication in the host cells (Wagner et al., 2002). Viruses with reduced sialic acid binding efficiency require less NA activity for viral release from the infected cells (Gubareva, 2004). Removal of the glycosylation site near the HA receptor binding pocket of A/Fowl plague virus (FPV)/Rostock/34 (H7N1) was reported to increase receptor binding affinity. This virus could only replicate well when matched with an NA of higher enzymatic activity. Compared to WH95-NA, UD72-NA had higher enzymatic activity, which enhanced replication of the WH95-HA N246S reassortant virus in MDCK cells and in the lungs of ferrets, suggesting that WH95-HA N246S might be more balanced with the UD-NA. It was interesting that the activity of CA04-NA was slightly higher than that of WH95-NA. The additional glycosylation sites in the globular head of CA04-HA is thought to decrease receptor binding activity and thus require lower NA activity for virus release. Additional H3N2 viruses, including A/Wisconsin/67/2005 (WI05) and A/Perth/16/2009 (Perth09) that have the same number of glycosylation sites in the HA as CA04, were examined and found to have lower enzymatic activities than CA04-NA (data not shown). These findings confirmed the general assumption that H3N2 virus containing more glycosylation sites requires less NA activity. The higher enzymatic activity of CA04 is an unusual case, which may have resulted in imbalance in their HA and NA activities. Sequence analysis of the NA proteins of the H3N2 viruses studied here showed that the NA proteins of WH95, CA04, WI05 and Perth09 have an additional glycosylation site at residue 329 compared to the NA of HK68 or UD72. Whether this additional glycosylation site in the NA contributed to the lower NA activity remains to be determined.

Ferrets have been a useful model for studying human influenza viruses, but it also has its limitations (Cohen, 2012; van den Brand et al., 2012). The outcome of influenza virus infection in humans is dependent on a number of factors, such as preexisting immunity to the same or different subtype influenza viruses, host innate and adaptive responses etc. Whether the levels of viral replication in the upper and lower respiratory tract of ferrets correlate with disease severity in humans remains to be further investigated.

Materials and methods

Viruses, cells and infection

Wild type (*wt*) influenza A H3N2 viruses including A/Hong Kong/08/1968 (HK68), A/Udorn/307/1972 (UD72), A/Wuhan/359/1995 (WH95) and A/California/07/2004 (CA04) were obtained from the Centers for Disease Control and Prevention or American Type Culture Collection (ATCC, Manassas, MA). MDCK cells were obtained from the ATCC and maintained in minimal essential medium (MEM) with 5% fetal bovine serum (FBS) and seeded onto 6-well plates at 5×10^5 cells per well. After overnight incubation, the cell monolayers were washed with phosphate-buffered saline (PBS) and infected with virus at multiplicity of infectivity (MOI) of 5.0 or 0.01. After adsorption at room temperature for 1 h, the cell monolayers were washed three times with PBS and incubated with OPTI-MEM I medium (Invitrogen, Carlsbad, CA) supplemented with 1 μg of tolysulfonyl phenylalenyl chloromethyl ketone (TPCK)-treated trypsin per ml. Culture supernatants were collected at 8, 24, 48 and 72 h post-infection (p.i.) and virus titer was examined by fluorescent focus assay (FFA) in MDCK cells. The plaque assay was performed by infecting confluent MDCK monolayer cell cultures with the virus. After incubation 1 h at room temperature, cultures were overlaid with MEM containing 1% agarose in the presence of 1 μg/ml of trypsin for 3 days at 37 °C. Plaques were fixed with methanol and stained with chicken anti-influenza polyclonal antibody, followed by horseradish peroxidase (HRP)-conjugated goat anti-chicken antibody (Dako, Carpinteria, CA).

Fluorescent focus assay (FFA)

Virus infectivity was measured by FFA in MDCK cell monolayers cultured in 96-well plates. Virus was serially diluted

10-fold in assay block in MEM, and transferred to MDCK cell monolayers. After the plates were incubated at 37 °C under 5% CO_2 for 18–22 h, the medium was removed; the cells were fixed with 4% paraformaldehyde and stained with mouse anti-human influenza A (H3N2) monoclonal antibody (MAB146, Takara, Shiga, Japan), followed by fluorescent-conjugated rabbit anti-mouse (Invitrogen). The focus formation was viewed and quantified under fluorescent microscope. The virus titer was expressed as focus formation units (FFU/mL).

Generation of recombinant virus

Recombinant CA04 was generated by reverse genetics as previously described (Jin et al., 2003). The six CA04 internal protein gene segments were used as a backbone for generation of the reassortant viruses. Briefly, viral RNA from CA04 was reverse transcribed and amplified by polymerase chain reaction (PCR). The eight cDNAs corresponding to the viral gene segments of CA04 were cloned into pAD3000 vector. The HA and NA genes of UD72 and WH95 were also cloned into pAD3000. Mutations were introduced into the HA using the Quikchange Lightning site-directed mutagenesis kit (Agilent, Santa Clara, CA). Each plasmid was confirmed by sequencing. Viruses were amplified in the allantoic cavity of 10–11-day-old embryonated hen's eggs at 37 °C for 3 days (Charles River, Wilmington, MA). Recombinant viruses were confirmed by sequencing analysis.

Ferret studies

Eight to twelve-week-old male and female ferrets from Simonsen were housed in individually ventilated cages (IVC) (Tecniplast, Via I Maggio, Italy). After anesthetization, ferrets were intranasally inoculated with 7.0 $\log_{10}$ FFU of virus per 1.0 ml dose (0.5 ml/nostril). Three days after infection, ferrets assigned for replication studies were euthanized, and the lungs and nasal turbinates were harvested. Viral titers in the lungs and nasal turbinates were measured by the 50% egg infectious dose (EID_{50}) and expressed as EID_{50} per gram of tissue (EID_{50}/g). Ferrets assigned for the immunogenicity studies were infected as described above, sera were collected on day 28 p.i. and assayed for antibody titers by microneutralization assay. All procedures were performed in accordance with federal, state and Institutional guidelines in an AAALAC-accredited facility and the protocols were approved by MedImmune's Institutional Animal Care and Use Committee.

Microneutralization assay

Prior to serologic analysis, ferret sera were treated with receptor-destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan) which was reconstituted with 10 mL of PBS per vial. 0.1 mL serum was mixed with 0.15 mL RDE and incubated at 37 °C for 18 h and adjusted to a final dilution of 1:4 by adding 0.15 mL of 2% sodium citrate followed by incubation at 56 °C for 45 min. Serum was two-fold serially diluted in EMEM medium containing 1 μg/ml of TPCK-trypsin, incubated with 100 μl of virus containing 100 units of 50% tissue culture infectious dose ($TCID_{50}$) in 96-well U-bottom microplates for 1 h at 37 °C. The antiserum-virus mixtures were transferred to the MDCK cells in 96-well plates and incubated at 37 °C for 6 days. The cytopathic effect (CPE) was observed under a microscope and the neutralizing antibody titer was defined as the reciprocal of highest serum dilution that inhibited 50% CPE.

Virus purification

Virus propagated in 10-day-old embryonated hen's eggs was centrifuged at 2500 revolutions per minute (rpm) for 10 min to remove cell debris followed by centrifugation at 18,000 rpm for 18 h. The virus pellet was resuspended in PBS and purified by two rounds of gradient centrifugations, first on a gradient of 15–40% sucrose, then on a gradient of 15–60% sodium tartrate. The purified virus was concentrated by centrifuging at 25,000 rpm for 3 h and resuspended in PBS. The total protein content of purified viruses was determined using Micro BCA Protein Assay Reagent (Thermo Scientific Pierce, Rockford, IL). Five micrograms of purified virus was digested with PNGase (NEB) and subject to gel electrophoresis on 4–16% PAGE with SDS. The protein band was quantified using ImageQuant TL (GE Healthcare, Uppsala, Sweden).

Measurement of the neuraminidase activity

The NA activity was measured with 2′-(4-methyumbelleferyl)-α-D-N-acetylneuraminic acid (MUNAN) (Sigma, St. Louis, MO) as substrate. One microgram of purified virus was initially diluted to 50 μl with 0.1 M sodium acetate buffer (pH 5.5), followed by serially 2-fold dilution with the same buffer. The diluted mixtures were incubated with 50 μl of 0.625 mM MUN for 1 h at 37 °C. The reactions were stopped by adding 100 μl of 0.5 M glycine buffer. The fluorescence reading (λ_{em}=460 nm) of the released chromophore 4-methyumbelliferone was determined with Wallac 1420 VICTOR2 plate reader (PerkinElmer, Forster City, CA) and was expressed as viral NA activity.

The NA protein enzymatic activity was also measured by transient expression of NA plasmid in BSR-T7 cells using Lipofectamine 2000 (Invitrogen). Five μg plasmid DNA of NA in pAD3000 was co-transfected with 0.5 μg of a control plasmid encoding the Firefly luciferase (FF-Luc) gene in the pVITRO vector (InvivoGen, San Diego, CA). At 48 h post-transfection, DNA transfected cells were lysed in 250 μl of Cell Culture Lysis Reagent Buffer (Promega, Madison, WI) with one cycle of freezing and thawing. An aliquot of 40 μl of cell lysate was assessed using the Luciferase Reporter Assay System (Promega). The luminescent signal was measured using GloMax 96 well Microplate Luminometer (Promega) (λ_{em}=482 nm). Another aliquot of 50 μl was serially diluted with 50 μL of 0.1 M sodium acetate buffer (pH 5.5) for NA assay as described above. The NA activity was normalized by $OD_{460\ nm}$ (NA) divided by $OD_{482\ nm}$ (FF-Luc) and defined as relative NA activity.

Data analysis

The NetNGlyc 1.0 web-server (http://www.cbs.dtu.dk/services/NetNGl) was used to predict N-glycosylation sites (Asn-X-Ser/Thr, where X is any amino acid except Pro) on the HA sequence. Graphing and statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). A P value of ≤ 0.05 was considered significant.

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